1997-2012: Fifteen Years of Research on Peptide Lunasin

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1. Introduction

1.1. Chemopreventive role of food peptides

Cancer is a major killer in today's world accounting for around 13% of all deaths according to the World Health Organisation. It has been estimated that by 2020, approximately 17 million new cancer cases will be diagnosed, and 10 million cancer patients will die [1]. Epidemiological evidence has demonstrated that as many as 35% of these cancer cases may be related to dietary factors, and thus modifications of nutritional and lifestyle habits can prevent this disease [2]. Cell experiments, animal models and human trials have revealed that a large number of natural compounds present in the diet could lower cancer risk and even, sensitize tumor cells against anti-cancer therapies [3]. Therefore, knowledge on the effect of diet components on health will bring new opportunities for chemoprevention through intense alterations in diet regimens.

In the last few years, food proteins and peptides have become one group of nutraceuticals with demonstrated effects preventing the different stages of cancer, including initiation, promotion, and progression [4]. Certain advantages over alternative chemotherapy molecules, such as their high affinity, strong specificity for targets, low toxicity and good penetration of tissues, have made food proteins and peptides a new and promising anticancer strategy [5].

Protease inhibitors are found in plant tissues, particularly from legumes. One of the most extensively studied inhibitors in the field of carcinogenesis is the soybean derived Bowman-Birk protease inhibitor (BBI). It is a 71-amino acids polypeptides which chemopreventive properties have been demonstrated in both *in vitro* and *in vivo* systems [6]. As a result of this evidence, BBI acquired the status of "investigational new drug" from the Food and Drug Administration in 1992, and since then, large-scale human trials are being carried out to

evaluate its use as an anticarcinogenic agent in the form of BBI concentrate (BBIC) [7-9]. These studies have shown that BBIC is well-tolerated by the patients and led to promising results for prostate and oral carcinomas.

Milk contains a number of proteins and peptides exhibiting chemopreventive properties. As an example, lactoferrin is a well-known whey protein for its inhibitory action on cancer cells proliferation, as well as for its antimicrobial, anti-inflammatory and antioxidant abilities [10]. The protective effects of orally administered lactoferrin against chemically induced carcinogenesis, tumor growth, and/or metastasis have been demonstrated in an increasing number of animal model studies, thereby suggesting its great potential therapeutic use in cancer disease prevention and/or treatment. Lactoferricin is a cationic peptide produced by acid-pepsin hydrolysis of lactoferrin. Similarly to its source protein, lactoferricin has been demonstrated, by cell culture and animal models, to exert anticarcinogenic properties against different types of cancer, such as leukemia, colon, breast, and lung cancer, among others [11]. This peptide acts through cell proliferation inhibition, apoptosis induction, angiogenesis suppression, and modulation of protein expression involved in different carcinogenesis pathways.

Recent studies have identified and characterized, peptides derived from animal and vegetal sources as promising chemopreventive agents [12-14]. One of these peptides, called lunasin, was identified in soybean and other plants and legumes. Studies performed in the last five years have revealed lunasin's properties in both cell culture and animal models, making it a potential strategy for cancer prevention and/or therapy. The purpose of this chapter is to summarize the evidence reported since lunasin's discovery in 1997 on its possible benefits as a chemopreventive agent as well as its demonstrated mechanisms of action.

2. Lunasin: Discovery and beyond

Lunasin has been described as a 43-amino acid peptide encoded within the soybean 2S albumin. Its sequence is SKWQHQQDSCRKQLQGVNLTPCEKHIMEKIQGRGDDDDD DDDD, containing 9 Asp residues, and an Arg-Gly-Asp cell adhesion motif [15]. Lunasin was first identified in the soybean seed, with variable concentrations ranged from 0.5 to 8.1 mg lunasin/g seed [16,17]. This variation has been found to mainly depend on the soybean genotype, suggesting the possibility of selecting and breeding varieties of soybean with higher lunasin contents [16]. The stages of seed development have also been found to affect lunasin's concentration, and a notable increase occurs during seed maturation [18]. However, sprouting leads to a continuing decrease of lunasin with soaking time. Recent studies have revealed the influence on lunasin content of environmental factors, such as temperature, soil moisture and germination time, as well as of processing conditions [19-21].

Presence of lunasin has been demonstrated in commercial and pilot plant produced soybean products, including soy milk, infant formula, high protein soy shake, tofu, bean curd, soybean cake, tempeh, and su-jae (Table 1) [22,23]. Results from these studies establish the influence on lunasin concentration in the food products of different parameters, such as the

soy genotype, the environmental factors, the manufacturing process and the storage conditions. Thus, these parameters might be used to control the content of this bioactive peptide.

Type of sample	Composition-main ingredients	Lunasin (mg/100 g product)	Reference
Regular soymilk	Soybeans	15.7 ± 1.3	[22]
	Soybeans	12.3 ± 0.8	[22]
	Soybeans	11.8 ± 1.3	[22]
	Whole soybeans	9.3 ± 0.3	[23]
	Soybeans, soybean oil, soy lecithin	9.2 ± 1.7	[23]
	Soy flour, Stevia sweetened	7.0 ± 0.1	[23]
	Soy flour, Stevia sweetened	6.3 ± 0.2	[23]
	Whole soybeans, filtered water	7.9 ± 0.0	[23]
	Whole soybeans, filtered water	6.3 ± 0.1	[23]
	Whole soybeans, filtered water	6.1 ± 0.1	[23]
	Whole soybeans	6.0 ± 0.6	[23]
	Whole soybeans	2.2 ± 0.1	[23]
	Soybeans, soy lecithin	5.2 ± 0.7	[23]
	Soybeans, calcium fortified	1.8 ± 0.3	[23]
	Aqueous extract of soybeans	2.3 ± 0.4	[23]
Organic soymilk	Organic soybeans, malted wheat and barley extract	18.9 ± 2.6	[22]
	Organic soybeans, malted wheat and barley extract	14.2 ± 1.1	[22]
	Organic soybeans	13.8 ± 2.6	[22]
	Organic soybeans	14.4 ± 2.4	[22]
	Organic soybeans	14.7 ± 0.8	[22]
	Organic soybeans, rice syrup	13.7 ± 0.9	[22]
	Organic soybeans, soy protein isolate	13.9 ± 1.0	[22]
	Organic soybeans, malt syrup	18.3 ± 2.4	[22]
	Organic soybeans, barley extract	10.7 ± 0.8	[22]
	Whole organic soybeans, isoflavones	9.1 ± 0.1	[23]
	Organic soybeans	8.8 ± 0.0	[23]
	Organic soybeans, calcium fortified	8.2 ± 0.0	[23]
	Whole organic soybeans, calcium enriched	5.6 ± 0.7	[23]
	Whole organic soybeans, calcium enriched	5.4 ± 0.6	[23]
	Organic soybeans	3.8 ± 0.6	[23]
	Organic soybeans	2.9 ± 0.8	[23]
Soy formula	Corn syrup, soy protein isolate	4.1 ± 0.4	[22]
	Corn syrup, soy protein	2.8 ± 0.2	[22]
	Rice syrup, soy protein concentrate	1.5 ± 0.1	[22]
	Soy protein isolate, soy oil, iron fortified	8.9 ± 0.4	[23]

Type of sample	Composition-main ingredients	Lunasin (mg/100 g product)	Reference
	Soy protein isolate, soy oil, iron fortified	8.1 ± 0.4	[23]
	Soy protein isolate, iron fortified	7.3 ± 0.4	[23]
Soft Tofu	Soybeans	9.6 ± 0.9	[22]
Soft Tofu	Soybeans	7.3 ± 1.0	[22]
Silken Tofu Kinugoshi	Soybeans	9.6 ± 0.7	[22]
Silken Tofu	Soybeans	4.4 ± 0.5	[22]
Silken Tofu	Soybeans	3.7 ± 0.5	[22]
Medium firm Tofu	Soybeans	14.3 ± 1.8	[22]
Organic Medium firm Tofu	Soybeans	6.7 ± 1.3	[22]
Firm Tofu	Soybeans	3.5 ± 0.2	[22]
Extra firm tofu Chinese style	Soybeans	3.7 ± 0.1	[22]
Baked tofu	Soybeans, soy sauce (wheat)	5.5 ± 0.3	[22]
Fried tofu	Soybean, soybean oil, soy sauce	0.4 ± 0.1	[22]
Dry tofu	Soybeans	2.5 ± 0.3	[22]
Soy shake	Soy and milk, chocolate flavored	1.3 ± 0.01	[23]
	Soy, milk, vanilla flavored	1.3 ± 0.01	[23]
	Soy protein isolate, milk, dark chocolate flavored	2.0 ± 0.04	[23]
	Soy, sor protein isolate, chocolate flavored	3.6 ± 0.02	[23]
Organic tempeh	Soybeans, *Rhizopus oligosporus*	n.d.	[22]
	Soybeans, brown rice, *R. oligosporus*	8.2 ± 0.4	[22]
	Soybeans, flaxseed, brown rice, *R. oligosporus*	6.1 ± 0.4	[22]
	Soybeans, brown rice, *R. oligosporus*	n.d.	[22]
Marinated bean curd	Soybeans, soy sauce	9.5 ± 1.0	[22]
Soybean curd noodle	Soybeans	n.d.	[22]
Deep fried soybean cake	Soybeans, soybean oil	1.9 ± 0.3	[22]
Baked soybean cake	Soybeans, soy sauce, sesame oil	1.1 ± 0.2	[22]

Table 1. Type, composition, and lunasin content of soybean-derived foods

In search of natural sources of lunasin besides soybean, a first screening has been carried out using different beans, grains and herbal plants. Lunasin has been found in cereal grains known for its health effects, such as barley, wheat, and rye [24-27]. Several seeds of oriental herbal and medicinal plants have been analyzed, finding that lunasin is present in all of the *Solanaceae* family, except *L. Chinensis*, but not in any of the *Phaseolus* beans [28]. These findings suggested the presence of lunasin or lunasin-like peptides in other grains and plants. This peptide has been identified in *Amaranth*, a plant well-known and used by the Aztecs for its high nutritional value and its biological properties [29]. A recent study has

revealed the presence of lunasin in different *Lupinus* cultivated and wild species [30]. A more rigorous and systematic search of lunasin and lunasin homologues in different seeds should be need to carry out in order to establish a relation between the presence of this peptide and the taxonomic properties of the plants.

2.1. Bioavailability of lunasin

One of the properties of an ideal cancer preventive agent is that it can be taken orally. This means being able to survive degradation by gastrointestinal and serum proteinases and peptidases, and to reach the target organ or tissue in an active form. Simulation of gastrointestinal digestion of lunasin has demonstrated that, while synthetic pure lunasin is easily hydrolyzed by pepsin and pancreatin, lunasin in soy protein is resistant to the action of these enzymes. Bioavailability studies carried out with animals have confirmed the preliminary results obtained by *in vitro* analysis. First studies carried out in mice and rats fed lunasin-enriched soy protein found that 35% of ingested lunasin reaches the target tissues and organs in an intact and active form [17,28]. Lunasin from rye and barley have also shown stability towards pepsin and pancreatin *in vitro* digestion and the liver, kidney, and blood of rats fed with lunasin-enriched rye or barley, respectively, contained this peptide as detected by Western blot [26,27]. Naturally protease inhibitors, such as Bowman-Birk protease inhibitor and Kunitz trypsin inhibitor have been demonstrated to exert a protective effect on lunasin against digestion by gastrointestinal enzymes, playing this protection a key role in making lunasin bioavailable [31]. These authors reported that lunasin is bioavailable after its oral administration to mice, reaching different tissues, including lung, mammary gland, prostate, and brain, where this peptide might exert is chemopreventive effects. These authors also found that lunasin extracted from the blood and liver of lunasin-enriched soy flour-fed rats was bioactive and able to suppress foci formation in the same concentration as synthetic lunasin.

A clinical trial focused on evaluating lunasin's bioavailability has demonstrated that in healthy volunteer men, 4.5% of lunasin ingested in the form of soy protein reaches plasma [32]. Results from this study are relevant in supporting future clinical trials to demonstrate cancer preventive properties of lunasin.

3. Lunasin's role as chemopreventive peptide

Peptide lunasin has demonstrated to exert promising chemopreventive properties against different types of cancers by both cell culture and animal experiments (Table 2). First studies performed with mammalian cells revealed that lunasin did not affect their morphology and proliferation. However, this peptide acted preventing their transformation induced by chemical carcinogens-7,12-dimethylbenz[a]anthracene (DMBA) and 3-methylcholanthrene (MCA) [33,34], viral and ras-oncogenes [33,35,36]. These experiments made lunasin be considered a "watchdog" agent in the cell nucleus that once the transformation event occurs, it acts as a surrogate tumor suppressor that tightly binds to deacetylated core histones disrupting the balance between acetylation-deacetylation, which is perceived by the cell as abnormal and leads to cell death [37]. This first mechanism of action involving

histone acetylation inhibition is considered as one of the most important epigenetic modifications acting on signal transduction pathways involved in cancer development [38,39]. When the cells are in the steady-state conditions, the core H3 and H4 histones are mostly deacetylated, as a repressed state. When cells were treated with peptide lunasin and well-known deacetylase inhibitor sodium butyrate, histone acetylation was inhibited in C3H10T1/2 fibroblasts and breast cancer MCF-7 cells [33,36]. Furthermore, lunasin has been demonstrated to compete with different histone acetyltransferase enzymes (HATs), such as yGCN5 and PCAF, inhibiting the acetylation and repressing the cell cycle progression [24,25,28]. Recently, we have reported that lunasin is a potent inhibitor of histones H3 and H4 histone acetylation [40]. Lunasin's inhibitory activity was found to be higher than that demonstrated by other compounds, such as anacardic acid and curcumin, which chemopreventive properties have been already reported [41-43]. Studies focused on elucidating lunasin's structure-activity relationship establish that lunasin's sequence is essential for inhibiting H4 acetylation whereas poly-D sequence is the main active sequence responsible for H3 acetylation inhibition [40] (Table 3).

Although first studies only established lunasin's capacity to act when transformation process happens, studies performed in the last few years have demonstrated that this peptide also acts on established cancer cells lines. This activity against different types of cancer cell lines is summarized in this chapter. Moreover, results obtained from cancer animal models are also included.

3.1. Chemopreventive properties against breast cancer

With a prevalence of about 4.4 million women and a lethality rate of more than 410,000 cases per year, breast cancer is the most common cancer disease and the leading cause of death in women worldwide [44]. Based on the prevalence of estrogen receptors (ER) within the cell, breast cancer is categorized into the ER–positive type and the ER-negative type. About 70-80% of all breast cancers are estrogen sensitive and they are treated by conventional procedures including surgery, radiation chemotherapy, and estrogen analogues. However, ER-negative tumors are more aggressive and resistant to treatments [45,46]. Therefore, searching for new preventive and/or curative strategies for this type of breast cancer has centered the interest of current investigations.

3.1.1. Lunasin against breast cancer in vitro

Up to one third on breast cancers that are initially ER-independent become resistant to endocrine therapy during tumor progression [47]. Due to this emergence of hormone-resistance, it is necessary to search for alternative therapies. Lunasin has been demonstrated to inhibit cell proliferation in ER-negative breast cancer MDA-MB-231 cells in a dose-dependent manner, showing an IC_{50} value of 181 µM [48]. Studies carried out to establish a structure/activity relationship showed an IC_{50} value of 138 µM for the 21 amino acid sequence localized at the C-terminus of lunasin, thus being the main responsible for lunasin's inhibitory effect on breast cancer cells proliferation [40].

Cell line	Cell proliferation	Cell cycle	Apoptosis	Gene expression	Protein levels	Other effects	Reference
Breast cancer MDA-MB-231	Inhibition	Arrest in S-phase	Induction	CDC25A, Caspase 8, Ets2, Myc, Erbb2, PIK3R1 and JUN genes	cyclins D1, D3, CDK4 and CDK6	Synergisms with aspirin	[48, 40, 57]
Breast cancer MCF-7 treated with Na-butyrate	n.r.	n.r.	n.r.	n.r.	n.r.	Synergisms with anacardic acid; HAT activity, inhibition of H3 and H4 acetylation and RB phosphorylation	[24, 25, 28, 33]
NIH3T3 treated with Na-butyrate	n.r.	n.r.	n.r.	n.r.	n.r.	HAT activity, inhibition of H3 and H4 acetylation and RB phosphorylation	[24, 25, 28, 33]
NIH3T3 transfected with viral E1A oncogen	n.r.	n.r.	n.r.	n.r.	protein p21	foci formation	[24, 25]
NIH3T3 transfected with ras-oncogenes	n.r.	n.r.	n.r.	n.r.	n.r.	colony formation, inhibition H3 acetylation	[36]
DMBA-induced C3H10T1/2	Inhibition	n.r.	n.r.	n.r.	n.r.	foci formation	[33]
MCA-induced C3H10T1/2	Inhibition	n.r.	n.r.	n.r.	n.r.	foci formation	[33]
DMBA-induced NIH3T3	Inhibition	n.r.	n.r.	n.r.	n.r.	foci formation; Synergisms with aspirin and anacardic acid	[33, 54]
MCA-induced NIH3T3	Inhibition	n.r.	n.r.	n.r.	n.r.	foci formation; Synergisms with aspirin and anacardic acid	[33, 54]
Colon cancer HT-29 and KM12L4	Inhibition	Arrest in G2/M phase	Induction	Modulation of Bcl-2, Bax, nCLU, cytochrome c and caspase-3; ↓ integrin 2, matrix metalloproteinase 10, selectin E, integrin 5 and collagen type VII α1 genes	↑ proteins p21 and p27	↑ activity of caspase-9	[59, 61]
Leukemia L1210	Inhibition	Arrest in G2 phase	Induction	n.r.	n.r.	caspase-8, -9 and -3 activity	[64]
Prostate epithelial RWPE-1	n.r.	n.r.	n.r.	HIF1A, PRKAR1A, TOB1, and THBS1 genes	n.r.	H4-Lys 8 acetylation; H4-Lys 16 acetylation	[66]
Macrophages RAW 264.7	n.r.	n.r.	n.r.	LPS-induced production of IL-1, TNF-, and NO genes; clusterin gene	IL-1, NO, and TNF-	COX-2/PGE2 and inducible iNOS/nitric oxide pathways; LPS-induced production of ROS; generation of hydroxyl radicals	[69-71, 73, 76]

n.r.: effect not reported

Table 2. Biological effects of peptide lunasin demonstrated by cell culture experiments

Lunasin fragment	Peptide sequence	Activity	Reference
f(1-43)	SKWQHQQDSCRKQLQGVNLTPCEKHIMEKIQGRGDDDDDDD	Inhibition of H4 acetylation (Lys8;Lys12;Lys5,8,12,16) Inhibition of H3 acetylation (Lys9; Lys9,14) Anti-inflammation	[40, 69]
f(1-22)	SKWQHQQDSCRKQLQGVNLTPC	Unknown or null function	
f(23-35)	EKHIMEKIQGRGD EKHIMEKIQGRGDDDDDDDD	Targets lunasin to histones Inhibition of H4 acetylation (Lys12; Lys5,8,12,16)	[40]
f(23-43)	RGD	A recognition site for integrin receptors present in the extracellular matrix, Internalize lunasin into cells	
f(34-43)	DDDDDDDDD	Directly binds to core histones Inhibition of H4 acetylation (Lys8; Lys12; Lys5,8,12,16) Inhibition of H3 acetylation (Lys9; Lys9,14)	[33, 40]

Table 3. Structure/activity relationship of lunasin and its derived fragments

A plethora of chromatin alterations appears to be responsible for the development and progression of various types of cancers, including breast cancer. Acetylation of specific lysine residues in histones is generally linked to chromatin disruption and transcriptional activation of genes [49]. In our studies, a dose-dependent inhibitory effect on H4 acetylation at positions H4-Lys8 and H4-Lys12 was observed after treatment of lunasin at 75 μM in MDA-MB-231 cells, reaching 17% and 19% inhibition, respectively, compared to control [40]. It should be needed to extensively study the relevance of these results on lunasin's chemopreventive activity to provide data about its molecular mechanism of action on epigenetic alterations. It would be useful to define new prognostic markers and therapeutic targets.

We have also demonstrated that lunasin modulates expression of different genes and proteins involved in cell cycle, apoptosis and signal transduction [48]. A pivotal regulatory pathway determining rates of cell cycle transition from G1 to S phase is the cyclin/cyclin-dependent kinases (CDK)/p16/retinoblastoma protein (RB) pathway. Over-expression of cyclins D1 and D3 is one of the most frequent alterations present in breast tumors. Cyclins D interacts with CDK4 or CDK6 to form a catalytically active complex, which phosphorylates RB to free active E2F [50]. Inhibition of deregulated cell cycle progress in cancer cells is being considered an effective strategy to delay or halt tumor growth. Lunasin up-regulates RB gene expression [48], and inhibits RB phosphorylation [28], suggesting that both transcriptional and post-translational modifications may be responsible for its inhibitory effect on cancer cell cycle progression. Moreover, lunasin has been found to inhibit cell proliferation, arrest the cell cycle in the S phase in 45%, and provokes a down-regulatory effect on the mRNA levels of CDK2, CDK4, CDC25A, Caspase 8, and Ets2, Myc, Erbb2, AKT1, PIK3R1 and Jun signaling genes in MDA-MB-231 cells [48]. Also, lunasin's down-regulatory action on levels of proteins, such as cyclin D1, cyclin D3, CDK4 and CDK6, might also contribute on its breast cancer MDA-MB-231 cells cycle arrest effect [40]. The ability of lunasin to modulate expression of genes and proteins involved in cell cycle, apoptosis and signal transduction seems to play a relevant role in its properties against breast cancer. However, further research should be needed to elucidate the complete molecular and epigenetic mechanism of action in breast cancer.

3.1.2. Lunasin against breast cancer in vivo

Lunasin's role as chemopreventive agent against breast cancer has also been demonstrated in *in vivo* mouse models. Our first findings showed a relevant inhibitory effect of a lunasin-enriched diet on mammary tumors development in DMBA-induced SENCAR mice [34]. Tumor generation and tumor incidence were reduced by 38% and 25%, respectively, in the mice fed with lunasin-enriched diet (containing 0.23% lunasin) compared with control group. Moreover, the tumor sections obtained from the lunasin-enriched group showed slight stromal invasion and degree of morphological aggressiveness due to the effect of this peptide contained in the soy protein preparation. Park and co-works have reported that isoflavone-deprived soy peptides prevent DMBA-induced rat mammary tumorigenesis, as well as inhibit the growth of human breast cancer MCF-7 cells in a dose-dependent manner, and induce cell death [51]. Lunasin might be responsible for the effects reported by these authors.

A recent study has shown that lunasin reduces tumor incidence and generation in a xenograft mouse model using human breast cancer MDA-MB-231 cells [31]. Lunasin's inhibitory effect on the tumor weight and volume was also reported by these authors. In contrast, BBI showed no effect on tumor development. The tumor histological sections obtained from the lunasin-treated group showed cell proliferative inhibition and cell apoptosis induction. These first animal models consider lunasin as a new and promising alternative to prevent and/or treat breast cancer.

3.1.3. Lunasin's combinations as a novel strategy against breast cancer

Cancer chemotherapeutic strategies commonly require multiple agents to prevent and/or treat cancer because of its ability to achieve greater inhibitory effects on cancer cells with lower toxicity potential on normal cells [3]. In the last two decades, it has been recognized the aspirin's chemopreventive role against different types of cancer. However, aspirin use has been associated with undesirable side effects, peptic ulcer complications, particularly bleeding and mucosal injury [52,53]. Studies are searching new agents to be combined to aspirin, increasing its effectiveness or decreasing its side effects. Our findings revealed that lunasin potentiates aspirin's cell proliferation inhibitory and apoptosis inducing properties in MDA-MB-231 cells [48]. This combination regulates the genes expression encoding G1 and S-phase regulatory proteins and the extrinsic-apoptosis dependent pathway, at least partially, through synergistic down-regulatory effects were observed for ERBB2, AKT1, PIK3R1, FOS and JUN signaling genes. Moreover, additional studies have demonstrated that lunasin/aspirin combination inhibits foci formation and cell proliferation in chemical carcinogens DMBA and MCA induced-NIH/3T3 cells [54]. The effect was notably higher than that observed when compounds of the combination acted as a single agent.

Anacardic acid is a natural compound found in the shell of the cashew nut. It has been linked to anti-oxidative, anti-microbial, anti-inflammatory and anti-carcinogenic activities [55,56]. Our findings revealed that lunasin/anacardic acid combination arrests cell cycle in S-phase and induces apoptosis at higher levels than that observed when each compound is used individually. This combination also promotes the inhibition of ERBB2, AKT1, JUN and RAF1 signaling genes expression. Synergistic effects have also been observed when lunasin was combined with anacardic acid to treat breast cancer cells and chemical-induced fibroblast cells [57].

The safety and efficacy of chronic use of these combinations should be further tested in animal models and human studies to establish the optimal dose and duration of treatment. Moreover, studies derived from these findings about mechanisms of action of these lunasin's combinations would open a new vision in the development of novel therapies against breast cancer.

3.2. Lunasin's chemopreventive properties against colon cancer

Colon cancer is the second leading cause of cancer death in the Western world. The high incidence, morbidity and mortality of colon cancer make necessary the effective prevention

of this disease. In the last years, pathogenesis of colorectal cancer has been elucidated, giving the approach for development of new drugs to combat this malignancy. Accumulating studies have shown the capability of bioactive food components to modulate the risk of developing colon cancer [58]. Recently, lunasin's potential chemopreventive role has been also reported.

3.2.1. Lunasin against colon cancer in vitro

It has been demonstrated that lunasin causes cytotoxicity in four different human colon cancer cell lines, KM12L4, RKO, HCT-116, and HT-29 cell, with IC_{50} values of 13.0 μM, 21.6 μM, 26.3 μM and 61.7 μM, respectively [59]. These values suggest that lunasin is most potent killing the highly metastatic KM12L4 colon cancer cells than any other colon cell lines used in this study. Moreover, lunasin was capable to provoke cytotoxic effects on the oxaliplatin-resistant variants of these colon cancer cells [60]. Studies on mechanism of action of this peptide have revealed that lunasin causes arrest of cell cycle in G2/M phase and induction of the mitochondrial pathway of apoptosis. The cell cycle arrest was attributed with concomitant increase in the expression of the p21 protein in HT-29 colon cancer cells, while both p21 and p27 protein expressions were up-regulated by lunasin treatment in KM12L4 colon cancer cells [59,61]. Moreover, treatment with lunasin decreased the ratio of Bcl-2:Bax by up-regulating the expression of the pro-apoptotic Bax and down-regulating the expression of the anti-apoptotic Bcl-2, also increasing the activity of caspase-3 [61]. This might be attributed to the increase in the expression of the pro-apoptotic form of clusterin which is positively affected by the increase p21 expression in cell nucleus. Treatment of lunasin causes translocation of Bax into the mitochondrial membrane resulting in the release of cytochrome c and the increase of the expression of cytosolic cytochrome c in KM12L4 cells. It was also demonstrated that treatment with lunasin provokes an increase in the activity of caspase-9 and caspase-3 in both HT-29 and KM12L4 cells [59]. Furthermore, lunasin has been showed to modify the expression of human extracellular matrix and adhesion genes [59]. The Arg-Gly-Asp motif present in the lunasin structure is a recognition site for integrin receptors present in the extracellular matrix (ECM). Integrins are heterodimeric receptors associated with cell adhesion, and cancer metastasis [62]. Treatment of KM12L4 cells with lunasin resulted in the modification on the expression of 62 genes associated with ECM and cell adhesion [59]. These authors also reported that lunasin down-regulated the gene expression of collagen type VII α1, integrin β2, matrix metalloproteinase 10, selectin E and integrin α5 by 10.1-, 8.2-, 7.7-, 6.5- and 5.0-fold, respectively, compared to the untreated colorectal cancer cells. On the other hand, the expression of collagen type XIV α1 was up-regulated upon lunasin treatment by 11.6-fold. These results suggest a potential role of peptide lunasin as an agent to combat metastatic colon cancer particularly in cases where resistance to chemotherapy develops.

3.2.2. Lunasin against colon cancer in vivo

Colon cancer liver metastasis is a widely used model to study the effects of different markers and chemotherapy on colon cancer metastasis. Recently, Dia & de Mejia (2011b)

have reported that lunasin acts as chemopreventive agent against this type of metastasis using colon cancer KM12L4 cells directly injected into the spleen of athymic mice [60]. Lunasin administered at concentration of 4 mg/kg body weight resulted in a significant inhibition of liver metastasis of colon cancer cells, potentially because of its binding to α5β1 integrin and subsequent suppression of FAK/ERK/NF-κB signalling. Lunasin was also capable to potentiate the effect of oxaliplatin in preventing the outgrowth of metastasis. Moreover, lunasin potentiated the effect of oxaliplatin in modifying expression of proteins involved in apoptosis and metastasis including Bax, Bcl-2, IKK-α and P65 [60]. These results suggest that lunasin can be used as a potential integrin antagonist thereby preventing the attachment and extravasation of colon cancer cells leading to its anti-metastatic effect. These results open a new vision about the lunasin used in metastasis that might benefit to prolong the survival of mice with metastatic colon cancer.

3.3. Lunasin's chemopreventive properties against other type of cancers

Leukemia is considered to be the most common type of cancer in children. Leukemia disrupts the normal reproduction and repair processes of white blood cells causing them to divide too quickly before they mature and resulting in the arrest on the proper production of all blood cells [63]. Chemopreventive properties of peptide lunasin have also been shown in human leukaemia L1210 cells, with an IC_{50} value of 14 μM [64]. Cell cycle analysis performed by these authors showed that lunasin caused a dose-dependent G2 cell cycle arrest and induction of apoptosis. The expressions of caspases-3, -8 and -9 were significantly up-regulated by 12-, 6- and 6-fold, respectively, which resulted in the increase of percentage of L1210 leukemia cells undergoing apoptosis from 2 to 40% [64].

Prostate cancer is one of the leading causes of cancer death in worldwide men. The multistage, genetic, and epigenetic alterations nature of prostate cancer during disease progression and the response to therapy, represent fundamental challenges in our quest to understand and control this prevalent disease [65]. Recently, Galvez and co-workers have studied lunasin's effects on tumorigenic RWPE-1 and non-tumorigenic RWPE-2 human prostate epithelial cells [66]. These authors observed that HIF1A, PRKAR1A, TOB1, and THBS1 genes were up-regulated by lunasin in RWPE-1 but not in RWPE-2 cells, confirming lunasin's capacity to selectively act on cancer cells without affecting non-cancerous cells. Moreover, lunasin specifically inhibited H4-Lys8 acetylation while enhanced H4-Lys16 acetylation catalyzed by HAT enzymes p300, PCAF, and HAT1A [66]. As a dietary peptide capable of up-regulate gene expression by specific epigenetic modifications of the human genome, lunasin is suggested to represent a novel food bioactive peptide with the potential to reduce cancer risk.

4. Anti-inflammatory and antioxidant activities of lunasin

Inflammation and oxidative stress are two of the most critical factors implicated in carcinogenesis and other degenerative disorders. Accumulating evidences have revealed that chronic inflammation is involved in the development of approximately 15–20% of

malignancies worldwide [67], being clearly associated with increased cancer risk and progression [68]. Lunasin has been found to exert anti-inflammatory activity that might contribute to its chemopreventive properties. First studies demonstrated that lunasin potently inhibits lipopolysaccharide (LPS)-induced production of pro-inflammatory mediators interleuquine-6 (IL-6), tumor necrosis factor-α (TNF-α), and prostaglandin (PG) E2 (PGE2) in macrophage RAW 264.7 cells [69], through modulation of cyclooxygenase-2 (COX-2)/PGE2 and inducible nitric oxide synthase/nitric oxide pathways, and suppression of NF-κB pathways [70,71]. Larkins and co-workers (2006) have demonstrated that COX-2 inhibition can decrease breast cancer cells motility, invasion and matrix metalloproteinase expression [72]. Abnormally up-regulated COX and PGs expression are features in human breast tumors, thus lunasin might have a role in treatment and prevention of this kind of cancer. Moreover, the same biological activity was observed for lunasin-like peptides purified from defatted soybean flour by combination of ion-exchange chromatography and size exclusion chromatography. These peptides showed potent anti-inflammatory activity by inhibiting LPS-induced RAW 264.7 cells through suppression of NF-κB pathways [70,71]. Interestingly, Liu and Pan (2010) used *E. coli* as a host to produce valuable bioactive lunasin that was also showed its anti-inflammatory properties. The purified recombinant lunasin form *E.coli* expressed system inhibits histone acetylation, and inhibits the production of pro-inflammatory cytokines, such as TNF-α, interleukin-1β and nitric oxide in LPS-stimulated RAW 264.7 cells [73].

Large amounts of reactive oxygen species (ROS) have been shown to participate in the etiology of several human degenerative diseases, including inflammation, cardiovascular and neurodegenerative disorders, and cancer [74]. It is believed that persistent inflammatory cells recruitment, repeated generation of ROS and pro-inflammatory mediators, as well as continued proliferation of genomically unstable cells contribute to neoplasic transformation and ultimately result in tumor invasion and metastasis [75]. Restoration/activation of improperly working or repressed antioxidant machinery or suppression of abnormally amplified inflammatory signaling can provide important strategies for chemoprevention.

Lunasin has been found to exert potent antioxidant properties, inhibiting linoleic acid oxidation and acting as a potent free radical scavenger, and reducing LPS-induced production of ROS by RAW 264.7 macrophage cells at a dose-dependent manner [69]. Recently, lunasin purified from *Solanum nigrum L.* has been found to protect DNA from oxidative damage by scavenging the generation of hydroxyl radical, as well as reducing Fe^{3+} to Fe^{2+} through blocking fenton reaction and inhibiting linoleic acid oxidation [76]. Moreover, these authors demonstrate lunasin's suppresive effects on the production of intracellular ROS and glutathione. Preliminary results indicate a similar inhibitory effect of ROS and GSH productions was also observed in Caco2 cells [77]. This activity might contribute on lunasin's chemopreventive role against cancer and other oxidative stress-related disorders.

5. Production of lunasin

Although the potential anticancer effect of lunasin has been demonstrated for over a decade, little progress has been made to test *in vivo* efficacy of purified lunasin in large-scale animal

studies or human clinical trials. The main limitations of these studies have been the lack of a method for obtaining gram quantities of highly purified lunasin from plant sources needed to perform such studies. Chemical synthesis is a rapid and effective method to produce lunasin in small quantities but the high cost and difficulties of the scale-up process makes lunasin's synthesis an economically impractical alternative. In addition, the process employs chemicals that are potential environmental hazards. To date, the reported methods to isolate and purify lunasin from soybean only allowed obtaining small quantities of this peptide at 80% purity [70]. However, recently, Cavazos and co-workers (2012) have developed an improved method to isolate and purify lunasin from defatted soy flour, resulting in at least 95% purity [23]. Simultaneously, a large-scale method to generate highly purified lunasin from defatted soy flour has been developed by Seber and co-workers (2012) [78]. This method is based on the sequential application of anion-exchange chromatography, ultrafiltration, and reversed-phase chromatography, obtaining preparations of > 99% purity with a yield of 442 mg/kg of defatted soy flour. Moreover, these preparations show the same biological activity than that reported for synthetic lunasin although the sequence contains Asn as an additional C-terminal amino acid residue.

An additional alternative to increase lunasin content in soybean has been recently reported [79]. This strategy aims to exploit the potential of sourdough lactic acid bacteria to release lunasin during fermentation of cereal and non conventional flours. After fermentation, lunasin from the water soluble extracts was increased up to 2-4 times, being *Lactobacillus curvatus* SAL33 and *Lactobacillus brevis* AM7 the strains capable to release higher concentrations of this peptide. This new strategy opens new possibilities for the biological synthesis and for the formulation of functional foods containing bioactive lunasin.

The use of recombinant production by transgenic organisms is widely employed in industry owing to their ease of use, robustness and costs, and has become the most effective system for the production of long peptides and proteins. A recent study has explored efficient recombinant production of lunasin by exploiting the *Clostridium thermocellum* CipB cellulose-binding domain as a fusion partner protein [80]. This system resulted in yields of peptide of up to 210 mg/L, but the authors consider that these yields might be increased in bioreactors where oxygen and nutrients levels are tightly regulated.

6. Conclusions

Peptides are becoming a group of health-promoting food components with promising chemopreventive and chemotherapeutic properties against cancer. Among them, peptide lunasin, found in soybean and other plants, is turning into one of the most promising. This peptide has been demonstrated its bioavailability after resisting gastrointestinal and serum degradation, and reaching blood and target organs in an intact and active form. Efficacy of lunasin against breast, colon, leukemia and prostate cancer using cell culture experiments and animal models have been revealed in the last decade. These results make lunasin a good candidate for a new generation of chemopreventive/chemotherapeutical agents derived

from natural seeds. However, there is still much to be learned about the effects and mechanisms of lunasin on cancer prevention/therapy. The major challenge on the use of lunasin in treating cancer would be the conversion of existing results into clinical outcomes. The next step should be to design clinical trials to confirm lunasin's chemopreventive properties against different types of cancer. Moreover, genomics, proteomics and biochemical tools should be applied to complete elucidate its molecular mechanism of action. Other aspects, such as searching for lunasin in other seeds, optimization of techniques to enrich products with this peptide and studying lunasin's interactions with other food constituents affecting its activity should also be conducted.

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